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Larvae of a Nudibranch Mollusc (*Phestilla sibogae*) Metamorphose when Exposed to Common Organic Solvents

J. TIMOTHY PENNINGTON[1] AND MICHAEL G. HADFIELD

Kewalo Marine Laboratory, University of Hawaii, 41 Ahui St., Honolulu, Hawaii 96813

Abstract. Larvae of the nudibranch mollusc *Phestilla sibogae* metamorphosed when exposed to 10 of 14 organic solvents. The active solvents included five alcohols and ethanolamine, acetonitrile, acetone, dichloromethane, and toluene. Inactive solvents were ethylene glycol, DMSO, benzene, and hexane. These compounds span a wide range of polarities and contain a number of functional groups. Ethanol induced metamorphosis after 1–5 days of exposure at 0.5–0.001 *M*, and maximally induced about 65% of larvae to metamorphose in 3–5 days at 0.1 *M*. Ethanol was lethal to larvae above 0.75 *M* (*ca.* 4%). Methanol was lethal only above 1.75 *M* (*ca.* 7%), but produced less metamorphosis than ethanol at most concentrations. The natural inducer of metamorphosis in *P. sibogae* produced higher percentages of metamorphosis more rapidly than did any of the solvents. The mechanism of metamorphic induction by the solvents is not known, but they probably interfere with a wide range of neuronal activities and trigger an existing metamorphic pathway. Precompetent (young) larvae did not metamorphose in response to ethanol or methanol, but juveniles produced by exposure of competent (mature) larvae to ethanol or methanol survived to reproduce. Larvae of one other mollusc species also metamorphosed in response to ethanol, suggesting that larvae of other invertebrates may also be induced to metamorphose by organic solvents. Larval biologists should be aware of this possibility.

Introduction

Many marine invertebrate species have complex life cycles, wherein a planktonic larval stage is both ecologically and morphologically distinct from the following benthic juvenile and adult stages. The planktonic and benthic segments of these life cycles are joined by relatively rapid and often drastic metamorphoses (see papers in Chia and Rice, 1978). Such metamorphoses have been subjected to considerable study (reviewed by Meadows and Campbell, 1972; Crisp, 1974, 1976; Burke, 1983), as researchers have been interested both in the ecology of metamorphosis and in the physiological and morphogenetic process of metamorphosis itself.

In nature, metamorphosis is initiated by environmental "cues" that are ecologically relevant; these cues induce larvae to metamorphose in sites where the probability of survival to adulthood is relatively high. For example, juveniles and adults of the nudibranch mollusc *Phestilla sibogae* Bergh eat only coral of the genus *Porites*, and larvae of *P. sibogae* metamorphose in response to a kairomone produced by these corals (Hadfield, 1977). However, pharmacological studies with *P. sibogae* and other invertebrates have revealed various neuroactive compounds that can also induce larvae to metamorphose (Hadfield, 1977, 1984; reviewed by Burke, 1983). These compounds are of no apparent ecological relevance, but interfere with larval nervous systems and apparently activate pre-existing metamorphic pathways. Such "artificial inducers" of metamorphosis have been useful as pharmacological probes during the study of the control of metamorphosis.

Workers in our laboratory have developed the *Phestilla:Porites* interaction as a system for the study of larval metamorphosis (reviewed by Hadfield and Pennington, in press). Here we present results of experiments with *P. sibogae* showing that organic solvents, and in particular ethanol and methanol, can serve as artificial inducers of metamorphosis. Additionally, we examine age-depen-

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[1] Present address: Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950.

dency of the response to ethanol and methanol, demonstrate that ethanol and methanol-metamorphosed nudibranchs survive to reproduce, and show that larvae of at least one other gastropod species also metamorphose in response to ethanol.

Materials and Methods

Larval culture

Routine larval culture methods for *Phestilla sibogae* have been fully described by Miller and Hadfield (1986). Briefly, embryos and early veliger larvae were raised at 25°C in their egg masses until 5 days old, when they were manually hatched. Hatched veligers were maintained in seawater with antibiotics until "competent" to metamorphose (see Hadfield, 1977, and below). Individual larvae of *P. sibogae* are either competent or not, but entire batches of larvae gradually become competent during days 7–9 of culture at 25°C.

Larvae of *Crucibulum spinosum* were provided by J. L. Bell (University of Hawaii). The hatched veligers had been fed in unstirred beakers (see Bell, 1988) for 21 days when they were tested for metamorphic response to ethanol.

Assays for metamorphic response to solvents

Most assays were conducted in soap-washed or acid-cleaned (Galigher and Kozloff, 1964, p. 25) 2-ml plastic tissue culture wells (Fisher Cat. No. 08-772-1), but where noted, assays were performed in acid-cleaned glass stender dishes. Both types of containers were covered during experiments. In experiments in culture wells, about 20 larvae were pipetted along with 200 μl of 0.22 μm-filtered seawater (FSW) into each of 2 replicate wells, each containing 1.8 ml of a given test solution. Test solutions consisted of organic solvents (analytical, HPLC, or photograde) dissolved in FSW, though where noted, a few comparisons employed MBL artificial seawater (Cavanaugh, 1956). The molar concentration of solvent in the final 2 ml of assay medium is reported. For those solvents relatively insoluble in water, a dilution series of solvent:seawater test solutions was vortexed and observed for disappearance of oily micelles. The most concentrated test solutions lacking persistent micelles were used to prepare assays of these solvents; 5 *M* stocks were used to prepare assays of solvents sufficiently soluble in water.

Larvae in the wells were counted and scored as metamorphosed or not on each of several day's exposure to solvent. Larvae were determined metamorphosed when they lost their vela and larval shells, thus becoming juvenile nudibranchs. All assays included both positive and negative controls. In negative controls, larvae were assayed as above in seawater alone, to control for any background or "spontaneous" metamorphosis. In positive controls, a living chip of *Porites compressa* (1–9 mm^3) was added to wells containing larvae and seawater to assess the competence of larvae to respond to the natural metamorphic inducer.

Several series of larval assays were conducted with *P. sibogae:*

(1) Detailed dose-response curves were constructed for both ethanol and methanol, spanning 5 orders-of-magnitude of alcohol concentration and 6 days of exposure, beginning with 11-day-old larvae. Six experiments of two replicate wells per concentration were conducted with each alcohol. Several of these experiments additionally compared responses in FSW *versus* those in MBL artificial seawater. Another experiment in this series compared responses to ethanol in acid-cleaned glass dishes and the plastic culture wells.

(2) In a second series of assays, 14 common organic solvents were surveyed for metamorphosis-inducing activity. These assays began with 11-day-old larvae and were run for 2 days over wide solvent concentrations (from lethal or near-saturated to no discernible effect).

(3) A third series of assays examined the effect of larval age (*i.e.*, precompetent-to-competent larvae) on responses to both ethanol and methanol. In these assays, larvae from the same culture but 6, 8, or 10 days old were exposed to coral, 0.5 *M* ethanol, 0.5 *M* methanol, or FSW alone and assayed for metamorphosis over the next 3 days. Four replicate wells of each of the alcohol treatments were used in this experiment.

Survival and growth of solvent-metamorphosed juveniles

An experiment was conducted to determine if juveniles of *P. sibogae* resulting from solvent-induced metamorphosis could survive and grow to adulthood. Eleven-day-old larvae were pipetted into culture wells containing 0.5 *M* ethanol, 0.75 *M* methanol, or 0.5 *M* methanol. Metamorphosed nudibranchs were removed from the solvent solutions over the next two days, counted, and transferred onto pieces of living coral. Juveniles from the different solvent solutions were placed on different pieces of coral, and each piece of coral was isolated in a small flow-through aquarium. The juveniles fed on the coral, grew, and became visible to the eye about 2 weeks after transfer. The nudibranchs were counted at this time and again at 24 days after transfer, when at least some in each basket had begun to lay eggs.

Response of Crucibulum spinosum *to ethanol*

Ten veligers of *C. spinosum* were pipetted into each of eight glass stender dishes. Two of the dishes contained 0.5 *M* ethanol in FSW, two contained 0.1 *M* ethanol in

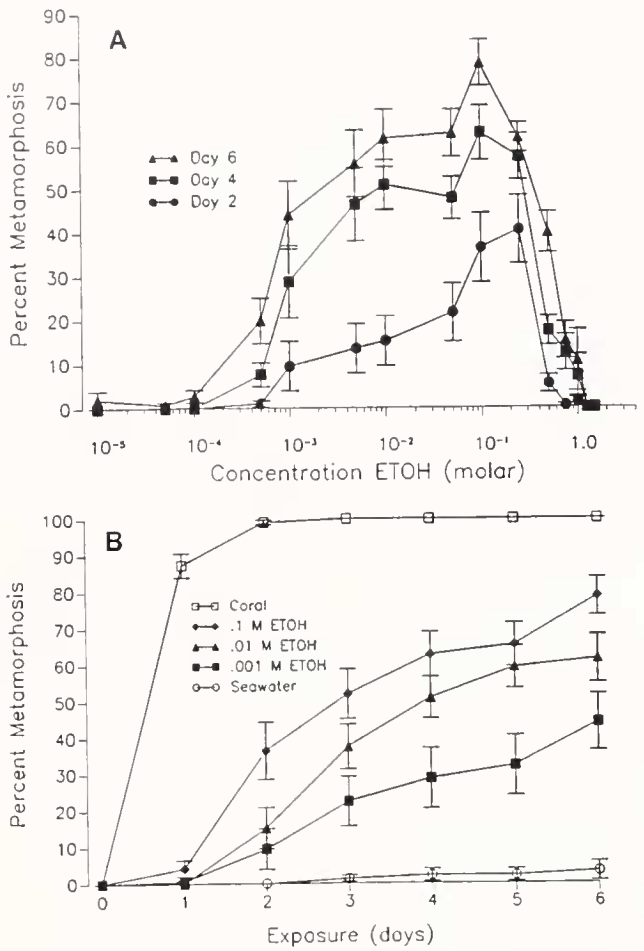

Figure 1. Cumulative percent metamorphosis of larvae of *Phestilla sibogae* exposed to a series of ethanol (ETOH) solutions for 6 days. Larvae were 11 days old at the beginning of experiments; plotted results are pooled means and S. D.'s of 6 experiments, each with 2 replicate assay wells per ETOH concentration, each well containing *ca.* 20 larvae. (A) Metamorphic response plotted as a function of ETOH concentration on days 2, 4 and 6; larvae were moribund and most eventually died at concentrations > 0.75 *M* (4%). (B) Response as a function of duration exposure to ETOH at 0.1, 0.01, and 0.001 *M*, maximal larval response was assayed with a coral chip, and "spontaneous" metamorphosis was monitored in wells containing larvae and seawater alone.

FSW, and two contained 0.01 *M* ethanol and FSW. The final pair of dishes contained FSW alone. The larvae were scored for metamorphosis on each of the next 3 days; animals were determined metamorphosed when their vela had completely disappeared.

Results

Assays for metamorphic response to solvents

Ethanol and methanol. Eleven-day-old veligers of *Phestilla sibogae* metamorphosed when exposed to both ethanol (Fig. 1A–B) and methanol (Fig. 2A–B). Very few of the larvae (<5%) metamorphosed over the 6 days of the experiments in filtered seawater alone, and 85–100% of the larvae metamorphosed within 1–2 days of exposure to a living chip of the coral *Porites compressa* (Figs. 1B, 2B). Maximal response to ethanol was between these control values, with 60–80% of larvae metamorphosing in 0.1 *M* ethanol (Fig. 1A). At least a few larvae metamorphosed at all doses between 5×10^{-4} and 1.0 *M*, but larvae were always moribund and usually died in solutions at and above 0.75 *M* (*ca.* 4%) ethanol. Maximal response to methanol was lower, with 20–35% of larvae metamorphosing in 0.1–1.0 *M* methanol (Fig. 2A). Effective doses ranged between 0.001 and 1.75 *M*, but a few metamorphoses occurred at lesser concentrations.

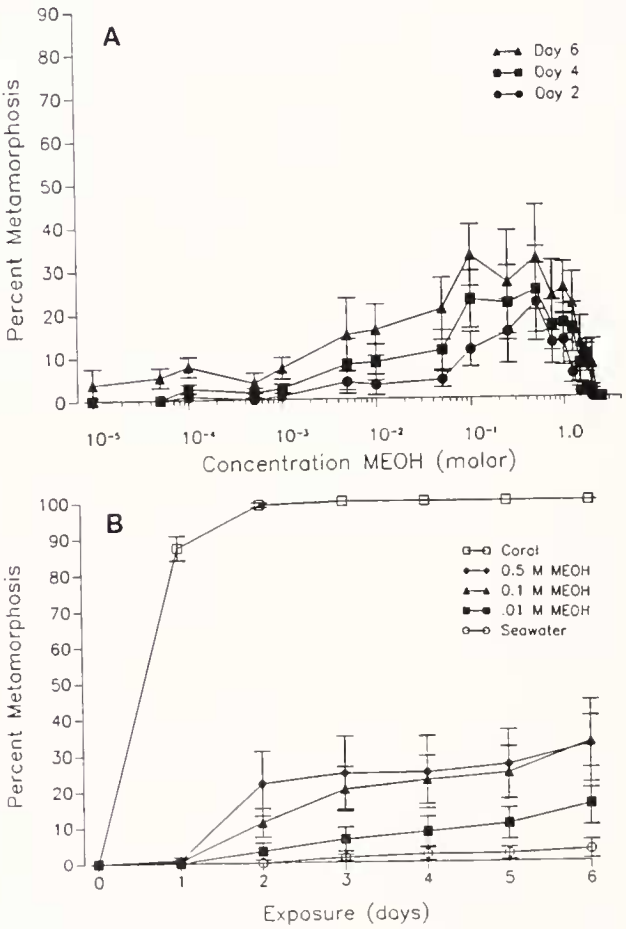

Figure 2. Cumulative percent metamorphosis of larvae of *Phestilla sibogae* exposed to a series of methanol (MEOH) solutions for 6 days. Larvae were 11 days old at the beginning of experiments; plotted results are as described under Figure 1. (A) Metamorphic response plotted as a function of MEOH concentration on Days 2, 4, and 6; larvae were moribund and most eventually died at concentrations > 1.75 *M* (7%). (B) Response as a function of duration exposure to MEOH at 0.5, 0.1, and 0.01 *M*; maximal larval response was assayed with a coral chip, and "spontaneous" metamorphosis was assayed in seawater alone.

Table I

Organic solvents assayed for capacity to induce metamorphosis of Phestilla sibogae, *listed by decreasing polarity*

Compound	Chemical family	Maximum percent metamorphosis [molar conc.]	Effective range (molar)
Ethylene glycol	diol	none —	—
Ethanolamine	amine	14 [0.01]	0.01
Methanol	alcohol	44 [0.5]	0.001–1.75
Ethanol	alcohol	39 [0.1]	0.0005–0.75
n-Propanol	alcohol	56 [0.05]	0.005–0.05
n-Butanol	alcohol	33 [0.001]	0.001–0.01
Acetonitrile	nitrile	35 [1.0]	0.1–1.0
DMSO	sulfoxide	none —	—
n-Pentanol	alcohol	18 [0.002]	0.0005–0.005
Acetone	ketone	83 [0.25]	0.05–1.0
Dichloromethane	halide	63 [0.3]	0.1–0.3*
Benzene	aromatic	3 [0.008]	0.008*
Toluene	aromatic	55 [0.003]	0.003*
Hexane(s)	alkane	none —	—

* Indicates near-saturation.

Larvae were moribund and usually died in methanol solutions above 1.75 *M* (*ca.* 7%).

The time-course of the response to both ethanol and methanol was much slower than to coral. Very few larvae metamorphosed in response to ethanol during the first day of the experiments, with increasing percentages of metamorphosis on succeeding days (Fig. 1B). Similarly, few larvae metamorphosed in methanol during the first day, with increasing percentages of metamorphosis over the second and third days (Fig. 2B). However, in methanol, few additional larvae metamorphosed after the third day.

Percentages of larvae metamorphosing in response to the alcohols in FSW or MBL artificial seawater were similar (data not shown). Experiments conducted in acid-cleaned glassware also produced similar percentages of metamorphosis to those in plastic culture wells. However, very few larvae metamorphosed in detergent-cleaned glass as compared to detergent-cleaned plastic or acid-eleaned ware of either material. Experiments were not conducted in detergent-cleaned glassware.

Other organic solvents. Including ethanol and methanol, 14 organic solvents were surveyed for their capacity to induce metamorphosis, and at least 10 solvents did so. The solvents are listed in Table I in order of decreasing polarity or water solubility, along with the general chemical family to which each belongs, the mean percent metamorphosis produced by the most effective concentration (molarity) of a solvent, and the effective ranges of concentration. Ethylene glycol, DMSO (dimethyl sulfoxide), and the hexane mixture did not produce any metamorphosis; the 3% metamorphosis observed in benzene is probably also questionable. However, the remaining solvents all produced metamorphosis in several replicate wells, and most did so at a number of concentrations. The highest solvent concentrations that did not kill or clearly incapacitate the larvae generally produced the most metamorphosis.

Response of precompetent veligers. Older, metamorphically competent larvae metamorphosed when exposed to either ethanol or methanol, but younger, precompetent larvae did not (Fig. 3A–C). When 6-day-old veligers were exposed to FSW or 0.5 *M* ethanol or methanol, essentially none metamorphosed over the next 3 days (days 6–9; Fig. 3A). At least some larvae became competent by days 8 and 9, as demonstrated by metamorphosis in the presence of coral, but few larvae metamorphosed in ethanol or methanol on these days, presumably because of the lag in response to alcohols as described above. When 8- or 10-day-old larvae were treated similarly, larvae began metamorphosing in both alcohols by day 10 (Fig. 3B) or day 11 (Fig. 3C). In these latter assays, no larvae metamorphosed in FSW alone, but at least some larvae were competent to do so during the first day of the experiments as demonstrated by the responses to coral (Fig. 3B–C). Again, larvae responded to these alcohols more slowly and less strongly than they did to coral.

Survival and growth of solvent-metamorphosed juveniles

When veligers were induced to metamorphose with ethanol or methanol and then grown on living coral, over 50% survived until at least some nudibranchs began to

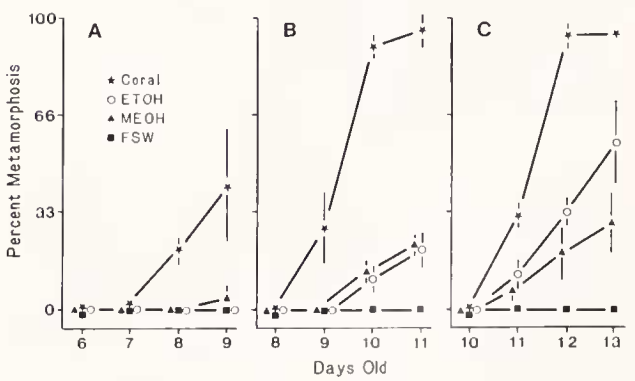

Figure 3. Cumulative percent metamorphosis of precompetent-to-competent larvae of *Phestilla sibogae* when exposed to either coral, 0.5 *M* ethanol (ETOH), 0.5 *M* methanol (MEOH), or filtered seawater (FSW) alone. Individual larvae are either metamorphically competent or not, but batches of larvae gradually become competent during days 7–9 at the culture temperatures used (25°C). Plotted results are means and S. D.'s of four replicate wells of each alcohol treatment and two replicates each of FSW and the coral treatment. (A) Response of 6-day-old, initially precompetent veligers. (B) Response of 8-day-old, mostly competent veligers. (C) Response of 10-day-old, fully competent veligers.

Table II

Phestilla sibogae: *survival of alcohol-induced nudibranchs until beginning of egg-laying*

Metamorphic inducer	Initial no. juveniles (Day 0)	Number alive (Day 14)	Number alive (Day 24)	Egg masses present? (Day 24)
Coral	10	4	3	yes
0.25 *M* ETOH	10	6	6	yes
0.5 *M* ETOH	4	4	4	yes
0.75 *M* MEOH	10	7	7	yes

lay eggs (Table II). It was usually not possible to determine which or how many of the nudibranchs in each basket had begun egg-laying. The observed survival rate was comparable to that observed when larvae were induced to metamorphose with coral (Table II), but there were no striking differences in survival among animals induced with the two ethanol doses or methanol. Nudibranchs in all treatments appeared normal. These results indicate that ethanol and methanol induce a true metamorphosis, producing juveniles that can grow to adulthood.

Response of Crucibulum spinosum *veligers to ethanol*

Veligers of *C. spinosum* metamorphosed by losing their vela when exposed to either 0.5 or 0.1 *M* ethanol for 1–3 days (Table III). No *C. spinosum* metamorphosed in either FSW or 0.01 *M* ethanol. Veligers in 0.5 *M* ethanol (*ca.* 1.5%) did not swim actively, as did larvae in the less concentrated ethanol solutions or FSW alone, but remained partly retracted or swam weakly at the bottom of the dish. J. L. Bell has successfully repeated this experiment several times (pers. comm.).

Discussion

A wide variety of common organic solvents induce metamorphosis of competent larvae of *Phestilla sibogae* (Table I). For ethanol and methanol, the response is a true metamorphosis, because precompetent larvae do not "metamorphose" in response to these alcohols (Fig. 3), and because competent larvae induced to metamorphose with ethanol or methanol can survive as juveniles and grow to adulthood (Table II). Other inducers of larval metamorphoses are suggested to be either (1) environmentally derived natural inducers, active at epidermal receptors on larval surfaces, (2) neurotransmitters, their analogues or their precursors, presumed to be active either at surface receptors or internally on larval nerves, or (3) cations thought to cause sensory cell or neuron depolarizations that activate existing metamorphic pathways (reviewed by Burke, 1983; Yool *et al.*, 1986). Because organic solvents do not normally occur in sufficient quantity to induce metamorphosis in natural seawater, are not normally involved in regulation of nerve function, and are not electrically charged, they cannot fall into any of these categories. Instead, the solvents probably penetrate larval tissues and interfere with a wide range of nervous activities, somehow activating a metamorphic pathway. This explanation is in general agreement with what is known of the pervasive and often narcotic effects of organic solvents on mammalian nervous systems (reviewed by Browning, 1965).

Because such a diversity of solvents successfully induced metamorphosis (Table I), specific functional groups of the solvent molecules are apparently not required for the induction. However, three of the solvents tested produced absolutely no metamorphosis, contrary to what might be expected if the simple presence of dissolved hydrocarbon was sufficient to induce metamorphosis. When the solvents were arrayed in order of decreasing polarity or water solubility (as in Table I), relationships between solvent polarity and maximum percent metamorphosis or effective solvent concentration were not apparent. Similarly, when arrayed by molecular weight (not shown), no obvious relationships between solvent molecular weight and percent metamorphosis or effective concentration were apparent. These simple pharmacological considerations do not clarify the means by which the active solvents induce larvae to metamorphose. Nevertheless, it was generally true that the highest concentrations of solvents that were not obviously toxic to larvae produced the highest percentages of metamorphosis.

Other artificial inducers of metamorphosis of *P. sibogae* include choline chloride (Hirata and Hadfield, 1986) and excess K^+ ions in seawater (Yool *et al.*, 1986). Additionally, epinephrine produces "partial metamorphosis," wherein veligers of *P. sibogae* lose their vela but not their shells (Hadfield, 1984). Choline maximally induces 60–70% of larvae to metamorphose at 3.75×10^{-3} *M*, after a 2–3 day latent period during which little metamorphosis occurs (Hirata and Hadfield, 1986). In con-

Table III

Ethanol-induced metamorphosis of Crucibulum spinosum

Metamorphic inducer	Percent metamorphosis		
	Day 1	Day 2	Day 3
Seawater	0	0	0
0.5 *M* ETOH	5	45	75
0.1 *M* ETOH	20	20	33
0.01 *M* ETOH	0	0	0

Values are means of 2 dishes containing 10 larvae each.

trast, live coral usually induces nearly all larvae to metamorphose within 48 h. The maximum percent metamorphosis produced by choline, the range of effective choline concentrations, and the delayed response to choline are all similar to our present results with organic solvents. Maximally effective choline doses are also just beneath toxic levels, as observed here with many of the solvents. These similarities might suggest that the solvents and choline function in the same or a similar manner. Conversely, the dose/response curves of the different solvents and choline are clearly different in some respects, and, unlike choline, the organic solvents cannot be involved in neurotransmitter biosynthesis (see Hirata and Hadfield, 1986).

Larval responses to both excess K^+ (Yool *et al.*, 1986) and epinephrine (Hadfield, 1984) are more rapid (*i.e.*, some response within 24 h) than to solvents or choline, and the maximum percentages of larvae to respond to K^+ are higher (>90%) than for solvents or choline. These differences probably indicate that solvents, K^+, and epinephrine operate via different mechanisms. Nevertheless, solvents and K^+, at least, probably induce metamorphosis through relatively nonspecific interference in nervous function.

We have had the opportunity to test larvae of only one additional invertebrate species for a metamorphic response to an organic solvent. In these experiments, larvae of the gastropod *Crucibulum spinosum* readily metamorphosed in response to ethanol. While these results are very limited, they suggest that competent larvae of other invertebrate species may also metamorphose in response to solvents. If so, ethanol in particular may prove of use to larval culturists as a widely available and relatively cheap and non-toxic inducer of metamorphosis. Conversely, in laboratories where organic solvents are commonly used, larval biologists should be aware that contamination of solutions by organic solvents may cause unwanted metamorphoses of competent larvae.

Acknowledgments

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